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13. ABSTRACT *(Maximum 200 words)*

With the support from the US Army Research Office, we have established the basis of using state-of-the-art atomic force microscope (AFM) to image biological specimens at sub-nm resolution at cryogenic temperatures under ambient pressure. Our instrumentation demonstrated convincingly that a contamination environment can be obtained as we proposed originally, and the performance of our prototype AFM at cryogenic temperatures was at least comparable to, if not better than, any atomic force microscope available today. We have also developed various methods to facilitate atomic force microscopy of biological specimens at room temperature. Modification of the Kleinschmidt methods enabled us to image DNA specimens in air at a resolution of 4-6 nm. Reliable preparation of supported bilayers enabled us to study the structure of lipid bilayers *in situ*, leading to the elucidation of interesting phenomena which are impossible to reveal by other available methods, and the imaging of membrane proteins in physiological conditions at a resolution of ~ 1 nm.. We also developed methods to image soluble proteins in solution at a resolution of ~ 1 nm. These suggest strongly the usefulness of AFM in biology that it can be used to solve problems which are difficult to tackle with other methods.

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Development and Application of Low-Temperature AFM

Final Report

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1. Introduction.

The advent of atomic force microscope in the late 80's has generated tremendous interests among biophysicists and biologists, because of the potential of the instrument. An AFM offers an unprecedented spatial resolution, and enables structural determination of biomacromolecules in physiological conditions. However, as we realized more than three years ago, two major problems must be solved in order to facilitate a wide application of AFM in biology. One is the softness of biological specimens. This is due to the contrast formation mechanism of the AFM, where the interacting force between a sharp tip and the specimen surface is responsible for image formation. Therefore, a finite probe force applies to the specimen, and, for biological specimens, tends to cause deformation or damage, degrading spatial resolution or even destroying the specimen. The other is the requirement to prepare appropriate specimens for AFM imaging. This is a general rule for any imaging instrument.

To overcome the serious drawback, the softness, of biological specimens, two approaches can be taken. One is to lower the probe force. For currently available AFM, a probe force as low as sub-nN ($< 10^{-9}$ Newton) can be used for stable imaging, and the instrument has been able to detect a force difference as small as $\sim 5 \times 10^{-11}$ Newton. Therefore, to rely on a significant improvement in lowering of the probe force may be technologically unrealistic even at present, not mentioning more than three years ago when we submitted the grant proposal to the US Army Research Office.

The other approach is to increase the mechanical strength of biological specimens. Since it has been known that biological materials increase their strength significantly at low temperatures, and that the structure of biomacromolecules can be preserved under a rapid freezing, we proposed to develop a low-temperature AFM system for ultrastructural studies of biological specimens more than three years ago. At cryogenic temperatures, the cryo-AFM images directly the surface of frozen specimens after freeze-fracturing and/or freeze-etching. Unlike with cryo-electron microscopy where the spatial resolution is limited by the finite size of shadowing heavy metal particles, a replica is no longer needed with cryo-AFM. Thus, sub-nm resolution imaging of biological specimens is possible, because this resolution is within the range of the instrument. However, since AFM is a surface-sensitive imaging instrument, it is particularly prone to contamination. Condensational contamination at cryogenic temperatures is especially serious. Surface-deposited contaminants could create image artifacts and interfere strongly with image interpretation. These problems were well thought in our proposal, and we proposed to solve them by operating an AFM under ambient pressure at liquid-nitrogen temperature. The cold nitrogen vapor from liquid nitrogen serves to purge out the system, to create a contamination-free environment before starting specimen manipulation and AFM imaging.

Besides to develop the low-temperature AFM for ultrastructural studies of biological specimens, we also proposed to use fully the potential of the AFM by developing methods to facilitate AFM imaging of biological specimens at room temperature. Although, sub-nm resolution was not conjectured possible at room temperature, useful information should still be obtained by imaging biological specimens at room temperature. AFM imaging at room temperature can evaluate specimens prepared for the low-temperature AFM. In particular, the capability to operate AFM in solution makes it possible to study the structure of biomacromolecules under physiological conditions, which

guarantees a natural and functional folding of proteins under study, leading possibilities to observe conformational changes in real time.

With the support from US Army Research Office, under the Funding Document DAAL03-92-G-0002, and the support from other funding agencies, we have been developing instrumentation and establishing several specimen-preparatory methods for biological applications of state-of-the-art atomic force microscopy (AFM). Among our accomplishments, we have proved experimentally that a contamination-free environment can indeed be obtained at cryogenic temperatures under ambient pressure. An AFM using optical signal-detection method was developed, and was operated at temperatures close to the liquid nitrogen temperature, with atomic resolution images of both mica and graphite obtained. Therefore, not only an appropriate imaging environment can be obtained, but also the performance of the AFM can be, at least, comparable to that of any currently available AFM. This guarantees the usefulness of a cryo-AFM for biological research. These establish a rational basis to develop methods for the preparation of frozen biological specimens to facilitate structural studies by cryo-AFM. Furthermore, Researchers in the biomedical community can also construct similar cryo-AFM for their studies, because of the availability of the technology.

We have also been developing several methods to facilitate AFM imaging of biological specimens at room temperature. In particular, we intended to use the capability of operating an AFM in solution for structural studies of membrane related problems, which are difficult for other available imaging instruments. Supported bilayers made of various lipids have been prepared in different physiological buffers. These allowed structural studies of model membranes, with interesting results obtained. Examples include Tris-induced ripple phase, and alcohol-induced interdigitation, on phospatidylcholine (PC) bilayers. Furthermore, supported bilayers served as matrices for structural studies of membrane proteins, with a resolution close to 1 nm obtained on membrane-bound cholera toxin B-oligomers.

Methods to allow *in situ* AFM imaging of soluble proteins were also established. Molecular resolution imaging of soluble proteins adsorbed on mica surfaces could be obtained routinely. In particular, we have achieved a resolution closed to 1 nm on pertussis toxin B-oligomers. Occasionally, features as small as 0.5 nm were clearly resolved on individual molecules from original AFM images without any data processing. Many of our successes took us by surprise. In the course of out investigation, we have also had better understanding about tip-sample interactions involved in AFM imaging. The effect of imaging environment, and the effect of tips were also investigated experimentally. Details of our results achieved in the past three years are given in Sec. 4.

2. Novel Instrumentation.

Although in our proposal a general framework was outlined that we intended to develop a low-temperature AFM system under ambient pressure, caution must be taken in achieving this goal. One can always start with a grand scheme, to design everything to the nuts and bolts before starting any construction. This would have been fine if one knew the final product were certain to work. For the low-temperature AFM to be developed in the proposal, we didn't have the luxury of knowing the final come out. The strategy we took was to develop a prototype initially, to test out the idea, and to get ourselves familiar with technical difficulties encountered. We started by designing a composite

prototype low-temperature AFM head unit. Therefore, the instrument would facilitate any later improvement by upgrading. This scheme proved to be beneficial.

To achieve the required criterion, a contamination-free environment, for imaging biological specimens at low temperatures, we analyzed existing problems in cryo-electron microscopy, and realized that to operate a low-temperature AFM under a low vacuum would be far from adequate. For ultra-high vacuum (UHV), a clean environment is possible. Yet, in the presence of biological specimens, which usually outgas intensively, the maintaining or even the obtaining of a UHV environment is of serious doubt. How can we get out of this dilemma? Since the majority of low-temperature contamination is from condensation, and the operation of the AFM does not require a vacuum environment, we examined the situation under ambient pressure. It was found that the presence of cold nitrogen gas should help to create a contamination-free environment, because nitrogen gas molecules at ambient pressure shorten effectively the mean-free-path length of H_2O molecules. Thus, for an enclosed system, after a sufficiently long settling time, environmental H_2O molecules should condensed into the liquid nitrogen. However, to facilitate remote control of a low-temperature AFM, it is likely that some gas leakage may appear. Of course one can try to prevent this kind of leakage by constructing very sophisticated mechanical devices as those used in UHV system. Is this necessary? We found that if a properly designed baffle assembly is attached to the top flange, the time for a molecule to reach the imaging surface diffusely is astronomically long. Here, the assumption was made that only the top flange would have required mechanical joints to facilitate communication between the AFM head unit and an outside operator.

Our conclusion drawn from these analyses indicated it quite reasonable and realistic to develop an ambient pressure AFM for imaging biological specimens. A natural question is how specimens are to be prepared? Decades of development in cryo-EM provided us with clues to prepare appropriate specimens for AFM imaging. Once an environment free from contamination can be established, frozen specimens can be freeze-fractured and/or freeze-etched, to expose biomacromolecules and/or macromolecular complexes for structural studies. Besides, ambient pressure operations of freeze-fracturing and/or freeze-etching do not introduce serious technical difficulties in addition to those encountered in similar operations for preparing cryo-EM specimens. Therefore, we established, at least in principle, that operating an AFM at low temperatures under ambient pressure should be suitable for structural studies of biological specimens.

By taking into account that biological materials become mechanically much stronger at low temperatures, a low temperature AFM should provide sub-nm resolution images of biomacromolecules and macromolecular complexes on frozen specimens after freeze-fracturing and/or freeze-etching, providing the instrument can deliver the same performance as it does at room temperature on hard surfaces, which is nothing but atomic resolution. This will open new avenues in structural biology. Important biological problems, such as identification of transmembrane domains, structural elucidation of translocation process in bacterial toxins, detection of conformation changes, just to name a few, will be within reach to be tackled.

3. *Developments of methods.*

At the time of our submitting this proposal, although we were clear that development of a low-temperature AFM would be of significant importance, we were also aware of the necessity to explore room temperature atomic force microscopy on various biological specimens. One major problem back then was the lack of understanding of details about various interactions between specimens and AFM tips. Even at present, our understanding remains poor, despite a rapid development of the field involving tremendous amount of experimental explorations in a world wide scope. However, a general consensus has been reached among researchers in the field of biological AFM that methods must be developed to prepare appropriate specimens to use fully the potential of the AFM.

For room temperature applications, two classes of specimens are suitable for AFM imaging. Specimens of one class are for AFM imaging in air, and those of the other class are for AFM imaging in liquids. Imaging in air, specimens are usually dehydrated. In this case, whether the dehydration causes any change in conformation of the biomacromolecules should be examined carefully. It seems that for filamentous nucleic acids, dehydration does not affect structural studies. For proteins, especially for membrane proteins, dehydration should be avoided. One major problem with imaging in air is the existence of an adhesion force between the specimen and the tip, this force can amount to a few hundreds of nN in some cases. The origin of this adhesion force varies case by case, and a hydration layer on the specimen is believed to be one of the most common causes. The adhesion force pulls down the tip onto the specimen, causing deformation or even damage. Several approaches can be taken to reduce the adhesion force. Examples include: to prepare a specimen differently, to image under a controlled humidity, or a combination of both, for possible minimization of the adhesion force. One can also image in alcohol. In this case, the adhesion force due to the hydration layer can be eliminated completely.

The capability of imaging in solution is the most unique feature of AFM, since biomacromolecules can thus be studies at a resolution as high as about 1 nm in physiological conditions. However, appropriate specimen-preparatory methods are essential to obtain high resolution. For example, direct imaging on cell surfaces revealed only features of ~ 20 – 50 nm, not sufficient to resolve individual membrane proteins on the surface, although larger scale features, such as rearrangement or reorganization of cell membranes, the movements of cytofilaments, can still be detected. We have shown that supported membranes are essential for high resolution imaging of membrane proteins, and for structural studies of model membranes. For soluble proteins, a proper substrate and other conditions require optimization for high-resolution imaging without excessive aggregates.

In addition to developing specimen-preparatory methods, the tip condition and the tip material also play an important role for successful applications of AFM. Two basic criteria for tips are sharp and less interactive. The former is to minimize a tip-broadening effect. The latter is essential to reduce tip-sample interaction for less specimen deformation. At present, a reliable method has not been found that allows the production of AFM tips to satisfy both criteria above.

4. Results.

With the convincing rationale stated above (although some of the specimen-preparatory strategies were developed later from our experience), we set forth to develop the low-temperature AFM instrumentation, and to explore room temperature AFM on various biological specimens. Our achievements have established firmly the position of AFM in structural biology. At present, the instrument is ready to study biological specimens. Other achievements include: (1) New information on several membrane related structures were obtained with *in situ* AFM; (2) Methods have been established for high-resolution imaging of membrane proteins and soluble proteins in buffers; (3) The imaging of pertussis toxins in solution revealed a high-resolution structure that are distinctly different from the one deduced from X-ray crystallography, indicating functional implications of the in-solution folding of the protein; and (4) Establishing a practical method to treat/clean tips for routine high-resolution imaging of DNA in air. These have been accomplished at the University of Virginia. Details are in the following.

4.a. Low-temperature AFM instrumentation.

We started our developments of the low-temperature AFM by constructing a prototype low-temperature AFM to operate in a small cryogenic dewar. This allows a relatively fast recycling time. A baffle assembly attached to the top flange was made of brass plates, and porous Styrofoam was used to fill the space between plates. The additional foam provides a better thermal insulation and a more effective contamination buffer. Since the low-temperature AFM was designed to operate under ambient pressure, we could take advantage of the large cooling power delivered by the cold nitrogen gas which evaporated from the liquid nitrogen. Thus, the laser diode assembly was mounted directly onto the AFM head unit. No thermal drift due to the heating from the laser diode was detected. Through experiments, we found that a single glass lens in the laser diode assembly worked better for low-temperature operations. To allow remote control, a locking mechanism was attached to each adjustment screws. This allows remote adjusting of the laser beam orientation, the photodetector position, and the initial engagement. Furthermore, the ability of remote adjusting allows to compensate any differential thermal expansion as the temperature lowers. Thus, for system rigidity, a brass block was used, avoiding the use of the more expensive invar, an alloy with the least thermal expansion coefficient.

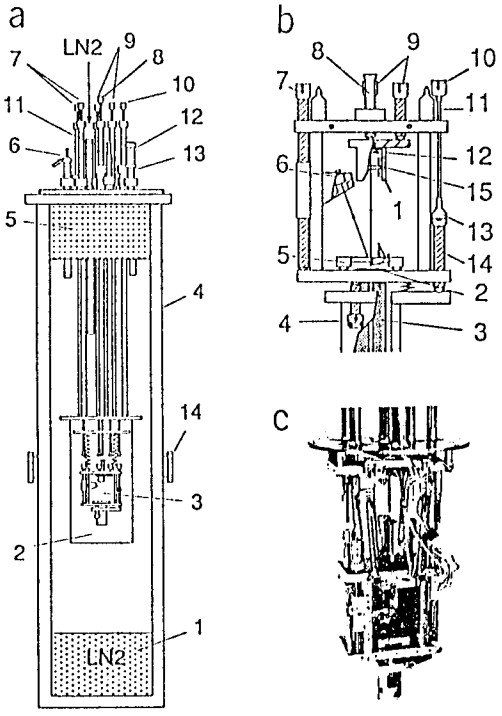

FIG. 1. The ambient pressure low temperature AFM in liquid nitrogen vapor: (a) illustration of the test system in a liquid nitrogen dewar: 1. Liquid nitrogen; 2. AFM chamber; 3. AFM head; 4. Liquid nitrogen dewar; 5. Baffle assembly with polyurethane foam; 6. Quick relief valve; 7. Photodiode position adjustments; 8. AFM head locking shaft; 9. Laser diode tilt adjustments; 10. Initial approach control; 11. Liquid nitrogen transfer port; 12. Electrical feedthrough; 13. Pressure relief valve; 14. View port; (b) Details of the AFM head design: 1. Laser diode assembly; 2. Sample holder; 3. Piezotube; 4. Coarse adjustment screws (3/16-100); 5. Cantilever holder; 6. Photodiode; 7. Photodiode position adjustment screws (1/4-80 vertical, 3/16-100 lateral); 8. AFM head lock; 9. Laser diode tilt adjustment screws (1/4-80); 10. Initial approach adjustment; 11. Alignment posts; 12. Laser diode; 13. Magnetic soft link; 14. Initial approach screw (3/16-100); 15. Focus lens; (c) The constructed low temperature AFM head, complete with the suspension and adjustment system.

Fig. 1 shows the prototype low-temperature AFM instrumentation and the head unit (adopted from the publication by Mou et al. 1993, listed in Sec. 5). The AFM head unit was quite rigid. With the dewar sitting on the fourth floor in a seven story building, atomic resolution images on hard crystalline surfaces, such as mica, graphite, and the 100 face of single crystals of NaCl, can be routinely obtained when the head was hung by four springs. In our low-temperature experiments, atomic resolution images of both mica and graphite were obtained, with the instrument running continuously for more than ten hours. Fig. 2 shows an image of HOPG at 79 K. This indicates a superb clean environment. Since otherwise, the contamination would have covered the surface to prevent any atomic resolution imaging. This is a markedly improvement over a previous report where an AFM was operated in a low-vacuum environment, and contamination prevented any meaningful surface imaging when the temperature was lowered only to about 150 K [Prater et al., 1991]. This experiment proved our conjecture in the proposal, and established the foundation for an upgrading of the system to image biological specimens.

One major upgrading to the low-temperature AFM instrumentation for bio-imaging is to incorporate specimen exchange devices. Thus, frozen specimens can be mounted at low temperatures to the AFM head. To facilitate this, a much larger cryogenic dewar was employed. The dewar was designed originally to incorporated an ambient pressure freeze-fracture/freeze-etch apparatus, a frozen specimen-storage place, and specimen-manipulation and specimen-transportation devices. As of present, a specimen transferring device has been installed, which allows exchange of specimens at low temperatures. This upgrading did not affect the rigidity of the system. Currently, we are still conducting experiments with the low-temperature AFM, and are designing and constructing other necessary accessory devices and apparatuses.

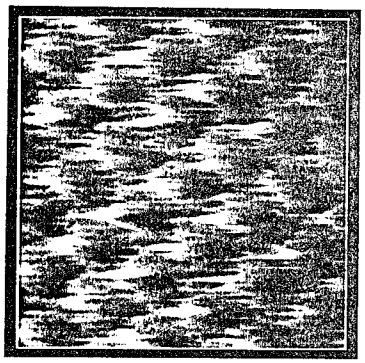

Fig. 2 HOPG image at 79 K.

4.b. Supported membranes.

The capability of operating in solution makes AFM particularly suitable for direct studies of the structure of membranes and membrane proteins. These studies have been very difficult with other methods. Using X-ray or electron diffraction, 3-D crystals are required. Up to present, not many successes have been reported to grow 3-D crystals of membranes. The growth of 3-D crystals of membrane proteins in the presence of lipid-bilayers has been even rare. With electron microscopy, the staining procedure treats the specimen so harshly that the integrity of the membrane is of serious doubt. Besides, the vertical resolution with electron microscopy is poorer than that can be obtained with the AFM. For light microscopy, the spatial resolution is far from revealing molecular details in the nm scale.

In the past three years, we have developed two major methods to prepare supported bilayers for *in situ* AFM imaging. Supported membranes are essential matrices for high-resolution structural studies of membrane proteins under physiological conditions, where a functional folding of the proteins is guaranteed. Since, on hydrated cell surfaces, the softness of cell membranes prevented resolving of

structural features any finer beyond ~ 20 - 50 nm, far from enough to resolve individual membrane proteins.

One of the methods is to use a Langmuir trough to prepare a lipid monolayer at the air/water interface. A substrate is immersed in the subphase solution initially before the formation of the monolayer. By withdrawing the substrate through the air/water interface, a monolayer is coated on the substrate. A bilayer is formed by immersing again the substrate into the subphase. The up and down strokes need to be slow for the formation of supported bilayers with a high percentage of surface coverage. The other method is to fuse directly lipid-vesicles onto a substrate to form a planar supported bilayer. With this method, the incubation duration and temperature are specimen-dependent parameters that need fine tuning for each individual cases. With both methods, supported bilayers made of phospholipids and lipid mixtures containing glycolipids were reliably prepared. Fig. 3 and Fig 4 are two examples of supported bilayers prepared by each method respectively.

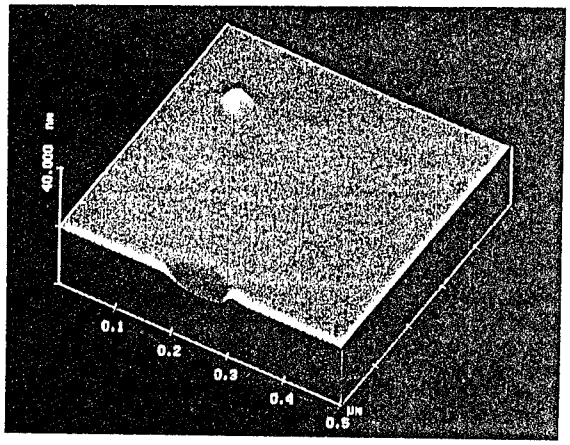

Fig. 3 DPPC bilayer prepared with a Langmuir trough.

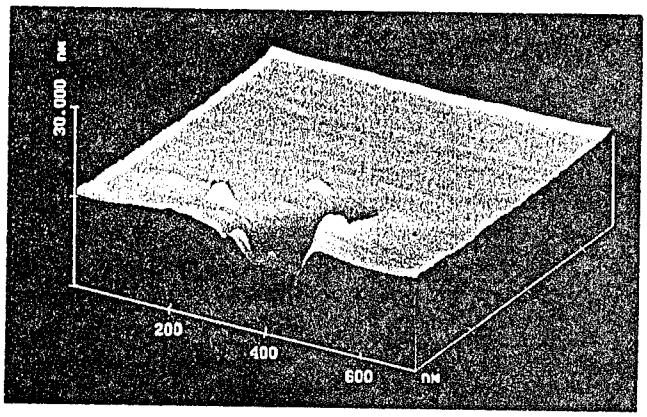

Fig. 4 DPPC bilayer prepared by the vesicle-fusion method.

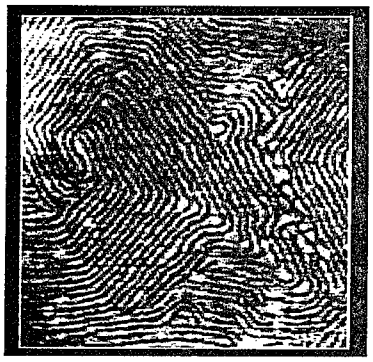

Fig. 5 Tris induce ripple phase in diC15-PC.
Image size: 1000 nm.

Fig. 6 Interdigitation domains in DPPC in 25% ethanol.
Image size: 1000 nm.

With supported bilayers, the structure of the membrane can be studied directly by *in situ* AFM. To our surprise, the commonly used Tris molecules were found to induce a ripple phase in phosphatidylcholine (PC) bilayers. Several interesting geometric patterns were observed, and they may indicate the packing symmetry of lipid molecules in the bilayer. Fig. 5 shows an example of Tris-induced ripple phase in a dipentadecanoyl-PC (diC15-PC) bilayer detected by AFM.

By incubating unilamellar PC bilayers with alcohol containing solutions, interdigitation of the bilayers was induced. Furthermore, it was found that interdigitated domains appeared at alcohol concentrations far below the threshold value well established by thermodynamic and diffractional studies. Fig. 6 shows an example.

These interesting results indicate strongly the suitability of *in situ* AFM for structural studies of bilayer membranes. Although details of the bilayer-formation process are not understood, nor do we understand how the Tris-molecules induce the ripple phase and why interdigitated domains existed at alcohol concentrations far below that required to induce a full interdigitation of the entire bilayer. To understand these, further studies are required. However, the establishment of bilayer-preparatory methods do indeed lay a solid foundation for structural studies of membrane proteins.

4.c. Membrane proteins: the cholera toxins and their B-oligomers.

Through experiments with red blood cells we have found a condition for reliable adhesion of the red blood cell to a glass coverslip, Fig. 7 shows a large scale red blood cell image obtained in a PBS solution. However, structural features any smaller than ~ 20 – 50 nm could not be resolved. This is due to the extreme softness of the cell surface when hydrated. Therefore, for structural studies of membrane proteins, a direct use of cell surfaces is not a valid approach. Realizing this, we set forth to develop methods to prepare supported membranes, with details already described above.

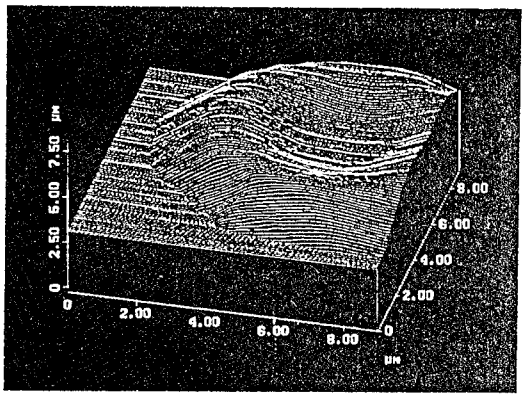

Fig. 7 A red blood cell in PBS buffer.

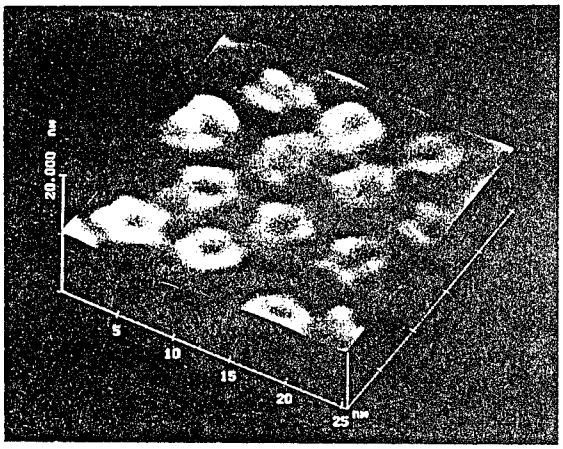

Fig. 8 Cholera toxin B-oligomers bound to a supported membrane of polymerizable phospholipids containing GM1.

The proteins we choose to demonstrate the validity of the method are cholera toxin and the cholera toxin B-oligomer. The structural determination of the cholera toxin was one of the objectives in our original proposal. Besides, extensive studies by other methods over past two decades have documented structural details of the cholera toxin. These would be extremely useful for identification of artifacts in image interpretation on AFM imagegraphes. Initially, polymerizable phospholipids were used to prepare supported bilayers. The next step was to prepare bilayers containing the cholera toxin receptor, the ganglioside GM1. Complete cholera toxins and cholera toxin B-oligomers were bound stably to the prepared supported bilayers. For randomly distributed membrane-bound cholera toxin B-oligomers, original AFM images without any image processing already showed many individual molecules having the representative pentameric structure. A resolution close to 1 nm was obtained. At this resolution, the identification of each individual molecules is of no problem (Fig. 8). Further, more details are revealed that allow the observation of subunit spatial arrangement of the B-oligomer. This is

a marked improvement over that in imaging a cell surface. A minor ambiguity, however, appeared when comparing the structures between the complete cholera toxin and the cholera toxin B-oligomer, where the former did not show a clear pentameric structure. The use of polymerizable lipids complicated image interpretation, a decisive conclusion was not reached regarding the different appearances.

Following further developments in methods to prepare reliable supported bilayers of non-polymerizable phospholipids, as described above, we came back to test the suitability of these more physiologically relevant bilayers for structural studies of membrane proteins. Indeed they do. Two examples are shown in Fig. 9 and Fig. 10. Fig. 9 shows cholera toxin B-oligomers on a bilayer made of dipalmitoyl-PC (DPPC) lipids containing 10 mol% GM1. Fig. 10 shows cholera toxin B-oligomers on a bilayer made of egg-PC containing 10 mol% GM1. The quality of the image is at least comparable to, if not better than, that obtained on the polymerizable lipids. One note that the egg-PC is a naturally extracting product that contains mixed acyl chains and is in a fluid-like state. The fluidity of the lipids does not affect the image quality. This opens doors to many other membrane proteins, they probably will be more at home on egg-PC mediated matrices, and can thus be studied *in situ* by AFM just as the case here with the cholera toxin B-oligomers.

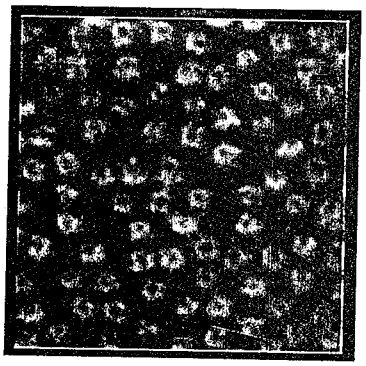

Fig. 9 Cholera toxin B-oligomers on DPPC:GM1. Image size: 80 nm.

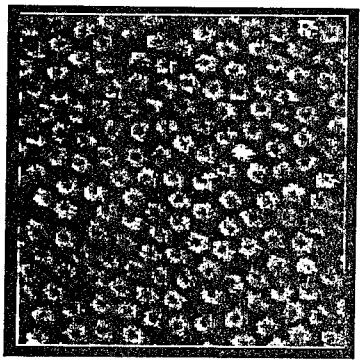

Fig. 10 Cholera toxin B-oligomers on egg-PC:GM1. Image size: 80 nm.

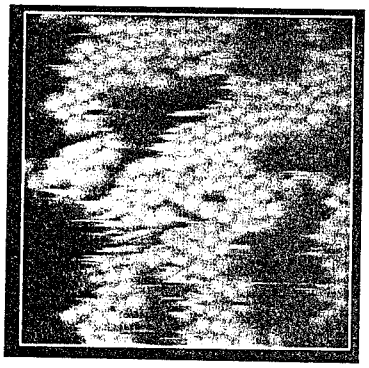

Fig. 11 Ferritin molecules in water Image size: 300 nm.

4.d. Methods to image soluble proteins in solution.

Soluble proteins are a large class of protein family. Many available methods allow structural determination of some of them. They include X-ray crystallography, electron microscopy, and NMR. The crystallographic method offers the highest 3-D atomic resolution. Yet, crystallization is specimen-dependent, and successful crystals have been grown on relatively small amount of proteins or protein subunits, considering the large number of soluble proteins available in the family. Electron microscopy requires to treat specimens harshly, by staining or fixing. Cryo-EM does preserve the structure. However, the shadowing particles limit the spatial resolution. NMR is limited to those proteins with a molecular weight not exceeding a few kD. Since AFM offers a high resolution imaging, a natural question is: can AFM be used to bridge some of the gaps in structural studies of soluble proteins? The presence of an adhesion force, and the dehydration that may result a denaturation of the soluble protein, rule out basically AFM imaging of soluble proteins in air. Therefore, it is essential to develop methods to facilitate AFM imaging of soluble proteins in solutions.

We studied carefully the adsorption of soluble proteins on mica for *in situ* AFM imaging, and found that the loosely bound proteins are the most problematic. They tend to attach to tips, causing an unstable adhesion force, sometimes as large as 10 nN, and creating image artifacts. One distinct example of the image artifact is the appearance of negatively contrasted images. The problem was solved by experimenting with different incubation conditions. The use of low protein concentration was also found beneficial. Fig. 11 shows an image of ferritin molecules on mica.

4.e. Structure and stability of pertussis toxin B-oligomers.

The methods we developed for imaging soluble proteins in solution have been used for structural studies of pertussis toxins. the pertussis toxin is a hexamer of 5 subunits (S1 - S5). The S1 (26 kD) is the catalytic subunit, responsible for intercellular action once translocated across the cell membrane. The rest five subunits form the receptor-binding B-oligomer. The B-oligomer consists of S2 (22 kD), S3 (22 kD), S5 (11 kD), and two copies of S4 (12kD). The difference in molecular weights of these subunits in the pertussis toxin B-oligomer indicates each of these subunits may have different roles in receptor binding and assisting the translocation. Therefore, it is important to determine the spatial arrangement of these subunits in the B-oligomer. In our studies, pertussis toxin B-oligomers adsorbed on mica surface were imaged by AFM in low salt buffers. Fig. 12a shows a typical image of pertussis toxin B-oligomers without any data processing. In many individual molecules, a clear 5-subunit structure can be seen directly. In order to obtain the information of spatial arrangement of the subunits, we used the program SPIDER to perform a correlative averaging on 300 individual molecules. The result is shown in Fig. 12b. It shows two larger units are next to each other. The implication of this result will be discussed shortly. One interesting observation is that, on many individual molecules, structural features as small as 0.5 nm were resolved clearly (Fig. 12c), indicating a remarkable resolution with *in situ* AFM imaging.

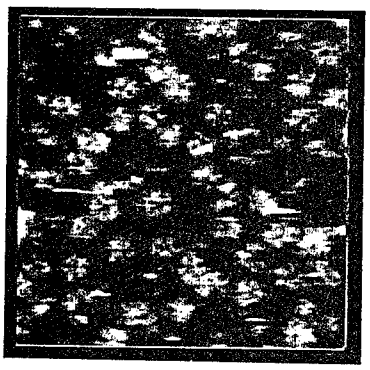

Fig. 12a Pertussis toxin B-oligomers mica. Image size: 65 nm.

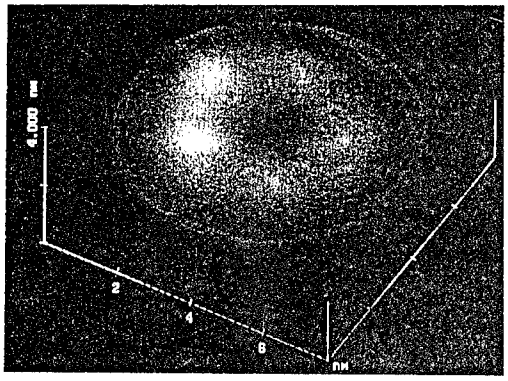

Fig. 12b Averaged B-oligomer.

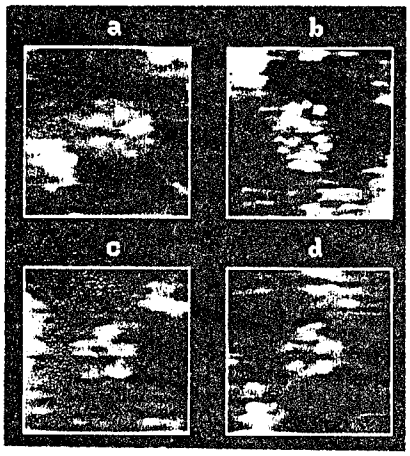

Fig. 12c High-resolution features of the on the B-oligomers. Image size for each: 15 nm

We also imaged the complete pertussis toxin. It was found most complete pertussis toxins have an overall molecular size similar to that of their B-oligomers. However, subunit structure was not resolved. We believe that the presence of the catalytic S1 subunit prevents the AFM tip from reaching onto the top of the subunits in the B-oligomer. As a result, only a globular structure was detected of the size of the molecule. We also studied the stability of the pertussis toxin B-oligomer under various temperatures and pH values. It was found that the structure of the B-oligomer remained intact for temperatures below 69 C, and for pH values in the range of 4.5 - 9.5.

Later, we were aware of a recent X-ray crystallography result [Stein et al., 1994] which came out the same time we put our work for publication. Comparing our structure with that obtained from X-ray diffraction on 3-D crystals, we found that there is a significant difference. In our case, most molecules show a five-unit structure, with the size of each unit having some difference. In the X-ray result, the B-oligomer is composed of seven domains, and these domains arrange themselves into a planar triangle. The size of each domain is about 4 nm. If the B-oligomer did indeed oriented themselves this way when adsorbing to mica surface in solution, this seven-domain structure would have been detected, since the resolution we achieved is more than enough to resolve the feature. Thus, what does this mean? A most likely explanation is that the B-oligomer folds differently in the two cases. In the 3-D crystals, the long range intermolecular interaction may cause the subunit to rearrange themselves to form the regular array. While in solution, more freedoms are available to allow spatial adjustment of the subunits in the B-oligomer. This is a unique case that such a distinct difference in structure is detected. It has been know that for small proteins, structural difference between X-ray studies and NMR imaging was detected [Billeter, 1992], and this difference enhanced for a relatively larger protein, the 74 residue α-bungarotoxin [Basus et al., 1988; Kosen et al., 1988]. The B-oligomer is of course a much larger protein, containing a total of 718 residues (234 for S1, 199 for S2 and S3, 110 for S4, and 100 for S5. B-oligomer contains S2, S3, S5, and two S4). Therefore, it is not entirely unreasonable to see the structure-difference. This may have significant functional implications. Further studies are needed to settle this issue.

4.f. Tip cleaning for routine high-resolution imaging of DNA in air.

The importance of tip-condition and tip-materials has been realized by researchers in the field. Yet, there has not been a single unique solution to this problem. We have shown that the commercially available Si_3N_4 tips delivered the same performance as the carbon-deposited "supertips". A careful examination showed that the advantage with those "supertips" and oxide-sharpened tips is that they gave a more consistent performance. For example, on a known test example, either a DNA specimen or a cholera toxin B-oligomer specimen, about 85% of "supertips" or oxide-sharpened tips tested, high-resolution images were obtained. With commercial tips, about 50% of them resulted in similar resolution.

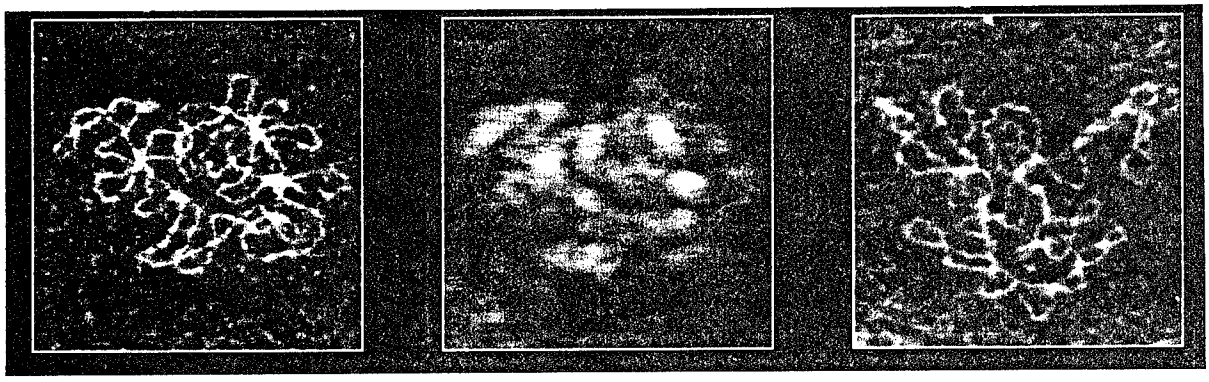

Fig. 13a: A 10 kb plasmid DNA imaged in air at an adhesion force about 2 nN. Image size for all: 400 nm.

Fig. 13b: The same DNA molecule when imaged at a larger adhesion force (~ 18 nN).

Fig. 13c: After tip cleaning, the resolution on DNA recovered.

In our experiments on imaging DNA with a modified Kleinschmidt method, we devised a method to clean tips. Thus, for normal Si_3N_4 tips, after the cleaning procedure, they can be used routinely for high-resolution imaging. The consistence is better than 90%. Fig. 13 shows the effect of this cleaning. Fig. 13a shows a 10 kb plasmid DNA imaged in air at an adhesion force about 2 nN. a resolution of 4-6 nm is obtained. Fig. 13b shows the same DNA molecule imaged at an adhesion force of about 18 nN. The degrading in resolution is apparent. Once a tip got into this state of performing so poorly, it could only at the best obtain images similar to the one shown here in Fig. 13b. However, after cleaning, the same tip could obtain high-resolution images again. Fig. 13c shows an example.

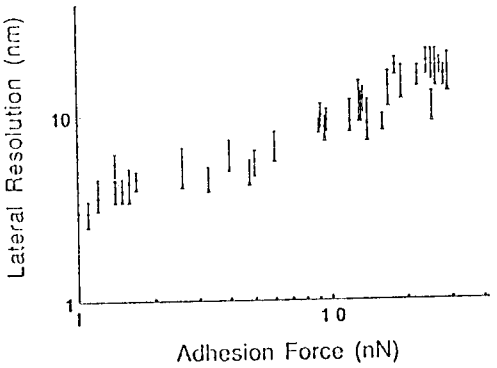

Fig. 14 Adhesion force *vs* lateral resolution.

The tip cleaning procedure we used was to scan a carbon-coated mica surface at line speeds of ~ 5 - 19 Hz, with an scanning area usually larger than 5 μm. There is an argument as to whether this procedure cleans the tip or picks up debris. The consistency of results, and the scrapping nature of scanning, strongly favor the former interpretation.

In our experiments, it is found that the adhesion force is a practical indicator about the tip condition. At larger adhesion forces, the resolution gets worse. A log-log plot of the relation between the adhesion force and the lateral resolution indicates a square root dependence, as shown in Fig. 14. This can be understood as the following. When a tip picks up some debris, its effective contact area increases. If the adhesion force is proportional to the contact area, a square root relation between the adhesion force and the resolution is established. This also implies that if a tip is made of a material that interact less strongly with the specimen, and hence is less likely to pick up debris, it should deliver a consistent high-resolution imaging.

4.g. Theoretical understandings of tip-sample interaction.

Considering what we have achieved, and an earlier theoretical estimation that a probe force of less than 10^{-11} N was required for biological applications of AFM, there is a significant gap between our understanding of the mechanism of AFM and our experimental results obtained with AFM. Bridging of this gap will also be essential for a full use of the potential of AFM. We started to analyze this problem by constructing a 2-D model, to study the effect of compression on resolution for a compressible sample through computer simulations. Initial results show that a finite compression actually leads to a better resolution, where the resolution is defined as the full width at the half-height. This is due to the nature of the localized interaction between the tip and the sample. Continuation of this study will provide a more rational basis for the understanding of the contrast formation mechanism in AFM imaging.

4.h. Summary.

We have established the basis for a low-temperature AFM to image biological specimens at cryogenic temperatures under ambient pressure, and have been developing the low-temperature AFM system for bio-imaging. We have also been developing various methods for biological applications of AFM at room temperature. Our accomplishments including: (1) Providing a contamination-free environment for cryogenic temperature AFM imaging; (2) Constructing the low-temperature AFM for biological applications; (3) Developing methods to prepare supported membranes; (4) Structural studies of model membranes with interesting results; (5) Establishing methods to study the structure of membrane proteins *in situ*; (6) Establishing methods to study the structure of soluble proteins *in situ*; (7) A resolution about 1 nm on membrane proteins and soluble proteins, leading to the revealing of new information on pertussis toxins; (8) Developing methods to prepare specimen and to clean tips for routine imaging of DNA molecules in air at a resolution of 4-6 nm; and (9) Starting a theoretical investigation of the contrast formation mechanism in AFM imaging through computer simulations. Our achievements, which would not have been possible without the support from the US Army Research Office, will lead to the use of AFM to solve important biological problems which are difficult to tackle with other available methods.

5. Publications and technical reports.

Articles:

1. Scanning tunneling microscopy of ionic crystals: ferritin core.
   Jie Yang, Kunio Takeyasu, Andrew P. Somlyo, and Zhifeng Shao, Ultramicroscopy, **45**, 199-203(1992).

2. Atomic force microscopy of DNA molecules.
   Jie Yang, Kunio Takeyasu and Zhifeng Shao, FEBS Letters **301**, 173-176 (1992).

3. A new approach for atomic force microscopy of membrane proteins: the imaging of cholera toxin.
   Jie Yang, Lukas K. Tamm, Thomas W. Tillack, and Zhifeng Shao, J. Mol. Biol., **229**, 286-290 (1993).

4. The effect of probe force on the resolution of atomic force microscopy of DNA.
   Jie Yang and Zhifeng Shao, Ultromicroscopy **50**, 157-170 (1993).

5. An Optical Detection Low Temperature Atomic force Microscope at Ambient Pressure for Biological Research.
   Jianxun Mou, Jie Yang and Zhifeng Shao, Rev. Sci. Instrum. **64**, 1483-1488 (1993).

6. Promises and Problems of Atomic Force Microscopy for Biological Research.
   Jie Yang, Lukas K. Tamm, Andrew P. Somlyo and Zhifeng Shao, Journal of microscopy, **171**, 183-198 (1993).

7. Resolution and Limitations in Biological Applications of AFM.
   Jie Yang, Lukas K. Tamm and Zhifeng Shao, in *Atomic Force Microscopy/Scanning Tunneling Microscopy*. S.H. Cohen, M.T. Bray and M.L. Lightbody, eds. in press.

8. Molecular Resolution Atomic Force Microscopy of Soluble Proteins in Solution.
   Jie Yang, Jianxun Mou and Zhifeng Shao, Biochim. Biophys. Acta **1199**, 105-114 (1994).

9. Structure and Stability of Pertussis Toxin Studied by *in situ* Atomic Force Microscopy.
   Jie Yang, Jianxun Mou and Zhifeng Shao, FEBS Letters **338**, 89-92 (1994).

10. Tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$) induced ripple phase in supported unilamellar phospholipid bilayers.
    Jianxun Mou, Jie Yang and Zhifeng Shao, Biochemistry **33**, 4439-4443 (1994).

11. Alcohol induces interdigitated domains in unilamellar phosphatidylcholine bilayers.
    Jianxun Mou, Jie Yang, C. Huang and Zhifeng Shao, Biochemistry **33**, 9981-9985 (1994).

12. Recent advances in biological atomic force microscopy.
Jie Yang and Zhifeng Shao, Micron, in press.

Invited Talks:

1. Cryo-temperature atomic force microscopy: instrumentation and application.
Jie Yang, Jianxun Mou and Zhifeng Shao, Proceedings of 52nd Annual Meeting, EMSA & 29th Annual Meeting, MAS, 414-415 (1994). (invited speaker: Jie Yang).

2. Atomic Force Microscopy of Phospholipid Membranes: From Ripples to Domains to Interdigitation.
Zhifeng Shao, Jie Yang, Jianxun Mou and Daniel Czajkowsky, Proceedings of 52nd Annual Meeting, EMSA & 29th Annual Meeting, MAS, 1050-1051 (1994). (invited speaker: Zhifeng Shao).

Abstracts:

1. BIOLOGICAL APPLICATIONS OF ATOMIC FORCE MICROSCOPY.
Jie Yang, Avril V. Somlyo, Michael K. Reedy, Kunio Takeyasu, Lukas K. Tamm, Margaretta Allietta, Thomas W. Tillack, and Zhifeng Shao, Proceedings of 50th Annual Meeting, EMSA & 27th Annual Meeting, MAS, 1138-1139 (1992).

2. Probe Force and Spatial Resolution in AFM of DNA.
Yang, J. and Shao, Z. [1993] Biophys. J. **64**, A10.

3. AFM of DNA, Membrane and Membrane Proteins.
Yang, J., Tamm, L.K., Tillack, T.W., Takeyasu, K. and Shao, Z. [1993] Biophys. J. **64**, A222.

Reports:

Technical Progress Report (11/1/91 - 12/31/91)

Technical Progress Report (1/1/92 - 6/30/92)

Technical Progress Report (7/1/92 - 12/31/92)

Technical Progress Report (1/1/93 - 12/31/93)

6. *Personnel*

Dr. Jianxun Mou, Postdoctoral Research Associate

Dr. Jie Yang, PI

7. Patent

1. Cryogenic Atomic Force Microscope.
   Andrew, P. Somlyo, Zhifeng Shao, Jianxun Mou and Jie Yang, US patent, approved.

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